Combined molecular biological and molecular cytogenetic analysis of genomic changes in 146 patients with B-cell chronic lymphocytic leukemia

A. BERKOVA[1*], L. PAVLISTOVA[1], L. BABICKA[1], L. HOUSKOVA[1], J. TAJTLOVA[1], P. BALAZI[4], E. CMUNT[2], J. SCHWARZ[3], J. KARBAN[2], M. TRNENY[2], J. BREZINOVA[3], Z. ZEMANOVA[1], K. MICHALOVA[1,2]

[1]Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, Faculty Hospital and First Faculty of Medicine, Charles University in Prague,U nemocnice 2,Praha 2 128 08 Czech Republic, e-mail: adelaberkova@yahoo.com; [2]First Medical Department, Faculty Hospital and First Faculty of Medicine, Charles University in Prague, Czech Republic; [3]Institute of Hematology and Blood Transfusion, Prague, Czech Republic; [4]Czech Technical University, Faculty of Nuclear Sciences and Physical Engineering, Prague, Czech Republic

Received February 2, 2008

Interphase fluorescence in situ hybridization was used to detect common deletions in B-CLL patients as well as trisomy 12 and aberrations of IgH gene complex at 14q32.33 where we evaluated not only translocation-like signal pattern but also deletions. 120 (82%) patients showed genetic changes – del(13)(q14) 95 (62%), deletion of ATM gene 22 (15%), deletion of p53 gene 25 (17%) and trisomy *12* was proved in 18 (12%) cases. IgH rearrangements were detected in 45 (31%), split of the signals in 11 (8%), deletion of 3´segment flanking IgH gene in 5 (3%) and deletions of variable segment in 29 (20%) patients. Although deletions of 3´segment flanking IgH gene complex are supposed to have an adverse prognostic impact and the genetic background of variable segment deletions is believed to be most probably physiological, we assumed a detailed mapping of the 14q32.33 region will be needed to unravel these mysteries.

Keywords: B-CLL; I-FISH; IgH gene complex deletions; prognosis

B-cell chronic lymphocytic leukemia (B-CLL) is the most common type of leukemia in adults in Europe and North America, where it takes up about 25 % of all leukemias in contrast to just about 5 % in the rest of the world. It was considered a clinically heterogeneous disease with a homogeneous cellular origin (i.e. immature, immune-competent, minimally self-renewing B cells [1]) which accumulate relentlessly because of a faulty apoptotic mechanism [2].

From a current viewpoint utilizing new information about the biology of leukemic lymphocytes published over the past decade, B-CLL is a disease derived from antigen-experienced mature B lymphocytes (monoclonal population of B cells in CLL expresses CD19, CD5 and CD23 and has reduced levels of membrane IgM, IgD and CD79b, a phenotype of mature, activated B lymphocytes) that differ in the level of immunoglobulin V-gene mutations [3]. Clinical courses of patients with B-CLL are profoundly disparate. Some survive for many years without treatment, whereas others have a rapidly fatal disease despite aggressive therapy. Clinical staging of B-CLL is based on two systems, described by Rai [4] and Binet [5], but neither one of these systems distinguishes at the time of diagnosis CLL patients, in which we can expect progression, from those with probable stable disease [6].

Within the past decade, many studies have searched for reliable prognostic markers able to predict prognosis and several so called risk factors have been assessed and statistically tested for relevance (e.g. genomic aberrations, ZAP-70 gene expression, IgV_H gene mutation status). The decision when and how to initialize the treatment remains critically important especially in younger patients with the presence of these risk factors and thus supposed (but not certain) more aggressive disease course.

Genetic analyses, among other modern diagnostic methods, have an important role in diagnosis, classification and management of patients with lymphoid malignancies. Unfortunately, conventional chromosomal studies are limited in CLL by the low number of mitotic neoplastic cells and

* Corresponding author

therefore normal results are obtained in most of the patients. Consequently, molecular cytogenetic method interphase fluorescence in situ hybridization (I-FISH) has become widely used to detect common recurrent deletions described in CLL patients such as *13q14, 11q22.3, 17p13.1, 6q21-23* as well as trisomy *12* [7, 8, 9, 10, 11].

At the Center of Oncocytogenetics, we routinely examine peripheral blood (PB) or bone marrow (BM) samples from B-CLL patients with a panel of DNA probes, detecting the most frequent genomic aberrations – trisomy *12, del(13)(q14.3), del(17)(p13.1)* and *del(11)(q22.3)*. With regard to remarkably frequent rearrangements of IgH gene (14q32.33) in B cell malignancies in general [12, 13] we included in examination of suspect B-CLL patients the LSI IGH break-apart rearrangement probe. We evaluated not only the break-apart signal pattern, but also deletions within IgH gene.

The aim of the presented study was: i) determining the frequency of chromosomal abnormalities, ii) to compare molecular cytogenetic results with disease status, ZAP-70 expression and IgV_H mutation pattern and iii) to evaluate the significance of IgH deletions and check whether these findings relate to a genomic localization of used VH gene segments stated by IgV_H mutation analysis.

Patients and methods

Cohort of Patients. In this retrospective study, PB or BM samples of altogether 146 patients with immunophenotypically confirmed B-CLL, were enrolled. 119 patients were examined at the time of diagnosis and the remaining 27, previously treated, at the time they relapsed.

I-FISH with all above named probes was evaluated in all cases. VH sequencing, ZAP-70 expression results and clinical data were available in 115, 91 and 98 cases respectively. The median age at diagnosis was 61 (range 32 – 83). The male to female ratio was 1.25 (81 men and 65 women).

Patients were classified into two groups according to the clinical course of the disease, in compliance with the commonly accepted NCI-WG criteria [14]:

1) with stable disease never requiring therapy – 52 patients (53%)
2) with progressive disease (developing progression or requiring therapy immediately) – 46 patients (47%) in presented cohort with available clinical data.

All patients in the disease relapse were assigned as progressive. Three deaths, all disease related, occurred within a median follow-up time of 37 months (range 5 to 221 months). The type of antileukemic therapy has not been monitored. All patients gave a written informed consent with the investigational procedures.

Sample Preparation for I-FISH. Bone marrow samples were cultivated for 24 hours in RPMI 1640 medium without mitogen stimulation and chromosomal slides were prepared according to standard procedures (0.075 KCl hypotonic treatment and fixation with Carnoy's fixative). Peripheral blood smear specimen were also prepared with conventional cytogenetic methods (hypotonic treatment of leukocytes and methanol – acetic acid 3:1 fixation). Cell suspensions were afterwards dropped onto microscopic slides and were directly used for I-FISH.

Analysis of Genomic Aberrations. All patients (146) were examined with all probes noted below. I-FISH analyses were performed on peripheral blood or bone marrow slides. Commercially available DNA probes from Abbott-Vysis™ (Vysis, Downers Grove, IL, USA) were used including following:

1. CLL Probe Panel allowing status assessment of these chromosome regions: *17p13.1* (gene p53), *11q22.3* (gene ATM), *13q14.3, 13q34* and *12p11.1-q11* (alpha satellite – centromeric – region of chromosome *12*)
2. LSI IGH break apart rearrangement probe designed to detect chromosomal breaks of the immunoglobulin heavy chain (IgH) locus, associated with *14q32.33* translocations; however, it does not identify the specific reciprocal translocation partner.

Two hundred nuclei were analyzed for each probe mix. The cut off levels for positive values were determined on samples obtained from 10 cytogenetically normal persons (5 male, 5 female) and were found to be 2.5% (mean ± SD).

ZAP-70 Expression Analysis by Flow Cytometry. Cytoplasmatic zeta-associated protein (ZAP-70) expression was measured in B lymphocytes from peripheral blood, collected in K_3EDTA, using the combination of three monoclonal antibodies: CD3 – FITC (fluorescein isothiocyanate) (Immunotech/Beckman Coulter, Fullerton, CA, USA)/ZAP-70 – PE (phycoerythrin) (Caltag/Invitrogen, Carlsbad, CA, USA)/CD-19 – PC-5 (phycoerythrincyanin-5) (Immunotech).

The IntraPrem™ Kit (Immunotech) was used for the essential cell permeabilization, enabling the antibody penetration into cells. The samples were analyzed by flow cytometer FACSCalibur (Becton-Dickinson, La Jolla, CA, USA) and evaluated by CELLQuest™ software. The results represent the percentage share of ZAP-70 positive B cells in the total B-lymphocyte count. ZAP-70 expression in less than 20% of cells was considered ZAP-70 negative whereas the cases exhibiting ZAP-70 expression in ≥20% of cells were interpreted as ZAP-70 positive.

Analyses of VH Mutation Status. The assay was performed in 120 cases, in some of them retrospectively, as the mutation status is deemed to be stable in CLL patients [15]. Ficoll-Paq isolated mononuclears were lyzed and RNA extracted using the TriZol reagent (Invitrogen, Carlsbad, CA, USA). RNA was transcribed into cDNA using Superscript II (Invitrogen), which was subjected to PCR amplification with Ampli-Taq Gold polymerase (Applied Biosystems, Foster City, CA, USA) in 6 separate assays to detect clonal proliferation in the 7 families of IgV_H genes. The touch down methodology using degenerated primers is described elsewhere [16].

RT-PCR products were purified and sequenced using the Big Dye Terminator Kit v. 3 and ABI Prism 310 Genetic Ana-

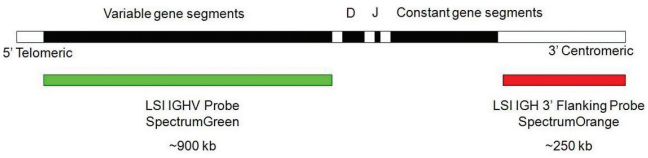

Figure 1. Abbott-Vysis™ LSI IGH Dual Color, Break Apart Rearrangement Probe. When hybridized to a normal nucleus, the probe produces two orange/green (yellow) fusion signal pattern.

Table I. Chromosomal aberrations found in 146 B-CLL patients.

del(13)(q14)	95/146 (65%)
del(13)(q14) incl. bial.	41
del(13)(q14) incl. bial., trisomy 12	5
del(13)(q14) incl. bial., del(17)(p13.1)	12
del(13)(q14) incl. bial., del(11)(q22.3)	8
del(13)(q14) incl. bial., 14q32.33 aberrations	16
del(13)(q14) incl. bial., trisomy 12, 14q32.33 aberration	1
del(13)(q14) incl. bial., del(17)(p13.1), 14q32.33 aberrations	4
del(13)(q14) incl. bial., del(11)(q22.3), 14q32.33 aberrations	7
del(13)(q14) incl. bial., trisomy 12, del(11)(q22.3), 14q32.33 aberration	1
trisomy 12	18/146 (12%)
trisomy 12	6
trisomy 12, del(13)(q14) incl. bial.	5
trisomy 12, del(17)(p13.1)	1
trisomy 12, 14q32.33 aberration	2
trisomy 12, del(17)(p13.1), 14q32.33 aberration	1
trisomy 12, del(11)(q22.3), 14q32.33 aberration	1
trisomy 12, del(13)(q14) incl. bial., 14q32.33 aberration	1
trisomy 12, del(13)(q14) incl. bial., del(11)(q22.3), 14q32.33 aberration	1
del(17)(p13.1)	25/146 (17%)
del(17)(p13.1)	6
del(17)(p13.1), del(13)(q14) incl. bial.	12
del(17)(p13.1), trisomy 12	1
del(17)(p13.1), 14q32.33 aberration	1
del(17)(p13.1), del(13)(q14) incl. bial., 14q32.33 aberrations	4
del(17)(p13.1), trisomy 12, 14q32.33 aberration	1
del(11)(q22.3)	22/146 (15%)
del(11)(q22.3)	2
del(11)(q22.3), del(13)(q14) incl. bial.	8
del(11)(q22.3), 14q32.33 aberrations	3
del(11)(q22.3), trisomy 12, 14q32.33 aberration	1
del(11)(q22.3), del(13)(q14) incl. bial., 14q32.33 aberrations	7
del(11)(q22.3), trisomy 12, del(13)(q14) incl. bial., 14q32.33 aberration	1
14q32.33 aberrations	45/146 (31%)
14q32.33 aberrations	8
14q32.33 aberrations, del(13)(q14) incl. bial.	16
14q32.33 aberrations, trisomy 12	2
14q32.33 aberration, del(17)(p13.1)	1
14q32.33 aberrations, del(11)(q22.3)	3
14q32.33 aberrations, del(13)(q14) incl. bial., del(17)(p13.1)	4
14q32.33 aberrations, del(13)(q14) incl. bial., del(11)(q22.3)	7
14q32.33 aberration, trisomy 12, del(17)(p13.1)	1
14q32.33 aberration, trisomy 12, del(11)(q22.3)	1
14q32.33 aberration, del(13)(q14) incl. bial., trisomy 12	1
14q32.33 aberration, trisomy 12, del(13)(q14) incl. bial., del(11)(q22.3)	1

incl. bial. – including biallelic

lyzer (both from Applied Biosystems). The sequences of IgV_H genes were compared to their germ-line configuration using the BLAST program (www.ncbi.nlm.nih.gov/igblast/) and a cutoff value of 2% (i.e. 98% of homology) was set to discriminate between the mutated and unmutated genes [17] (i.e. a sequence that differs from its germ line counterpart by 2 percent or more is defined as mutated).

Statistical Analyses. The dependence of various prognostic parameters was analyzed using the Mann-Whitney test (Table 3). Descriptive statistics are included in contingency tables. Overall survival was analyzed using the Kaplan-Mayer regression method and the statistical significance was calculated using the Breslow (Generalized Wilcoxon) test. All analyses were performed at the 95% confidence interval and the P values were found using SPSS software (version 15) [18].

Results

Of the 146 patients studied with I-FISH, 120 (82%) showed genetic abnormalities (in the extent of performed examination). Complete I-FISH results are summarized in Table I.

Deletion of *13q14* was proved in 95 (65%) patients, thereof in 41 (43%) patients as a sole abnormality. We have included besides monoallelic also biallelic deletions. In the remaining 54 cases, *del(13)(q14)* was combined with other chromosomal aberrations.

Trisomy *12* was observed in 18 (12%) cases, in 6 (33%) patients as the only genomic change investigated. Deletion of p53 gene at *17p13.1* and deletion of ATM gene at *11q22.3* were found in 25 (17%) and 22 (15%) patients, as a sole chromosomal change in 6 (24%) and 2 (9%) cases respectively.

Concerning the IgH locus (*14q32.33*) aberrations, we have detected these gene complex abnormalities in 45 (31%) cases from which in 8 (18%) patients as the only genomic alteration examined. The scheme of the probe used (LSI IGH from Abbott-Vysis™) illustrates Figure 1.

We have detected this high percentage of *14q32.33* changes due to the fact that this category comprises:

– deletion of variable segment of IgH gene i.e. loss of one green signal (found in 15 cases)
– partial deletion of variable segment of IgH gene i.e. one green signal apparently diminished (14 cases)
– deletion of 3´segment flanking IgH gene i.e. loss of one orange signal (5 cases)
– split of the probe signals indicating translocation (11 cases).

Comparison of particular I-FISH findings with disease and mutation status data, where available, is listed in Table II.

Statistical Data Evaluation. We have evaluated the available data from different aspects. In the first step we performed the standard non-parametric test [19] to see which variable significantly differs with respect to the progression of the disease. We have tested all available variables and used SPSS's two independent sample test and the method

chosen Mann-Whitney test. The results are summarized bellow (Table III).

It is obvious that the probability of progression significantly differs (on 0.05 significance level) for these variables: Rai stadium, age at diagnosis over 60 years, deletion of 13q, deletion of ATM, deletion of 3'flanking IGH gene (here is P = 0.063), ZAP 70 positive and mutational status mutated. It turns out that even the derived variables (deletion of p53 or deletion of ATM as a sole chromosomal abnormality) are also significantly differently distributed in patients with stable vs. progressive disease, whereas the other two variables, deletion/partial deletion of variable segment of IgH gene and deletion/partial deletion of variable segment of IgH gene/deletion of 3´segment flanking IgH gene, were detected within the progressive/stable group more or less equally (Table IV).

In the next phase, we have examined the distribution of times between the progressive vs. stable disease course observations with respect to various variables. We have used the Kaplan-Meier method of computing time-to-event models [20]. The Kaplan-Meier model is based on estimating conditional probabilities at each time point when an event occurs and taking the product limit of those probabilities to estimate the survival rate at each point in time.

Table II. Focus on IgH gene aberrations – number of patients with particular finding; clinical and mutational status data not complete.

deletion of IgH gene variable segment of	15
progressive disease	5
stable diasease	7
mutated	5
unmutated	8
germ line IgVH gene not identified	0
partial deletion of IgH gene variable segment	14
progressive disease	6
stable diasease	3
mutated	3
unmutated	7
germ line IgVH gene not identified	1
deletion of 3´segment flanking IgH gene	5
progressive disease	3
stable diasease	0
mutated	1
unmutated	1
germ line IgVH gene not identified	2
split indicating translocation	11
progressive disease	2
stable diasease	4
mutated	5
unmutated	3
germ line IgVH gene not identified	1

Table III. Statistical significance of component variables classified by disease status.

		Disease status				
		Stable		Progressive		
		No.	%	No.	%	Mann-Whitney P
Rai stadium	0	34	65,38%	3	6,67%	< 0,001
	1	14	26,92%	13	28,89%	
	2	4	7,69%	15	33,33%	
	3	0	0,00%	7	15,56%	
	4	0	0,00%	7	15,56%	
Female-0/Male-1	0	22	42,31%	21	45,65%	0,740
	1	30	57,69%	25	54,35%	
age >60	0	16	30,77%	23	50,00%	0,053
	1	36	69,23%	23	50,00%	
trisomy 12	0	46	88,46%	43	93,48%	0,393
	1	6	11,54%	3	6,52%	
del(13)(q14)	0	10	19,23%	25	54,35%	< 0,001
	1	42	80,77%	21	45,65%	
deletion of p53	0	44	84,62%	37	80,43%	0,587
	1	8	15,38%	9	19,57%	
deletion of ATM	0	48	92,31%	35	76,09%	0,027
	1	4	7,69%	11	23,91%	
deletion of variable segment of IgH gene	0	45	86,54%	41	89,13%	0,698
	1	7	13,46%	5	10,87%	
partial deletion of variable segment of IgH gene	0	49	94,23%	40	86,96%	0,216
	1	3	5,77%	6	13,04%	
deletion of 3´segment flanking IgH gene	0	52	100,00%	43	93,48%	0,063
	1	0	0,00%	3	6,52%	
split indicating translocation	0	48	92,31%	44	95,65%	0,493
	1	4	7,69%	2	4,35%	
ZAP-70 positivity	0	40	76,92%	18	47,37%	0,004
	1	12	23,08%	20	52,63%	
mutation status mutated	0	14	27,45%	29	70,73%	< 0,001
	1	37	72,55%	12	29,27%	
uncaptured used IgVH gene	0	48	94,12%	39	90,70%	0,531
	1	3	5,88%	4	9,30%	

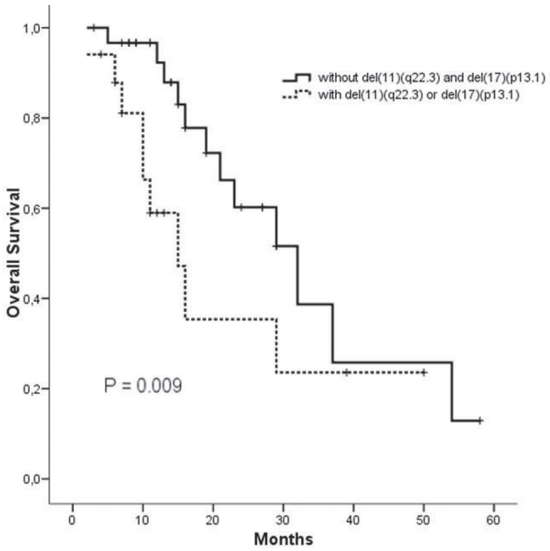

Figure 2. OS according to presence of deletion of ATM and/or p53.

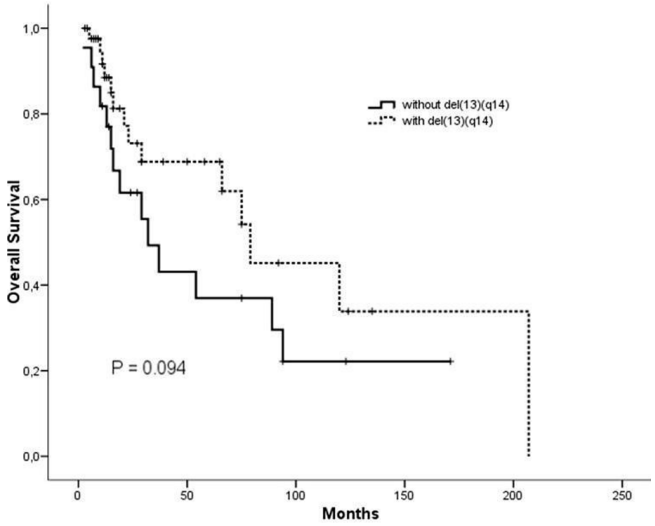

Figure 3. OS according to the presence of 13q14 deletion.

In presented figures 2, 3, 4, 5, the OS is the overall survival in months and the event measured is the disease status progressive vs. stable. All the P values are calculated on 0.05 significance level.

We have tested all parameters and identified 5 variables which indicate significant differences between the survival curves – Rai stage, ZAP-70 (not shown), mutation status, deletion of 13q14 and derived variable: deletion of p53 and/or deletion of ATM. The equality of survival times across groups was tested using the Breslow (Generalized Wilcoxon) test. Any point on the survival curve shows the probability whether a patient with a given property will/will not have experienced progression by that time.

In other words the probability that a patient without a deletion of ATM and/or p53 will not have a progressive disease is 0.7 after 20 months and 0.35 with a deletion of ATM and/or p53 (Figure 2). Further we proved that the patients with deletion of 13q14 have probability to develop a progressive disease 0.45 after 50 months compared to 0.7 in the patients without this deletion (Figure 3). Analogically, the probability that a patient will not have a progressive disease after 50 months is 0.75 for those whose mutation status is mutated and 0.4 for those who have unmutated IgV_H genes (Figure 4). The Kaplan-Meyer curve evaluating overall survival within individual Rai stage groups is depicted on Figure 5.

Discussion

In this study, we analyzed the association and prognostic impact of chromosomal aberrations and other parameters in 146 patients with B-CLL. From current aspect, we exam-

Table IV. Statistical significance of derived variables classified by disease status.

		Disease status				
		Stable		Progressive		
		No.	%	No.	%	Mann-Whitney P
deletion of p53 only	0	52	100,00%	42	91,30%	0,031
	1	0	0,00%	4	8,70%	
deletion of ATM only	0	52	100,00%	43	93,48%	0,063
	1	0	0,00%	3	6,52%	
deletion or partial deletion of variable segment of IgH gene	0	42	80,77%	35	76,09%	0,575
	1	10	19,23%	11	23,91%	
deletion or partial deletion of variable segment of IgH gene or deletion of 3´segment flanking IgH gene	0	42	80,77%	33	71,74%	0,295
	1	10	19,23%	13	28,26%	

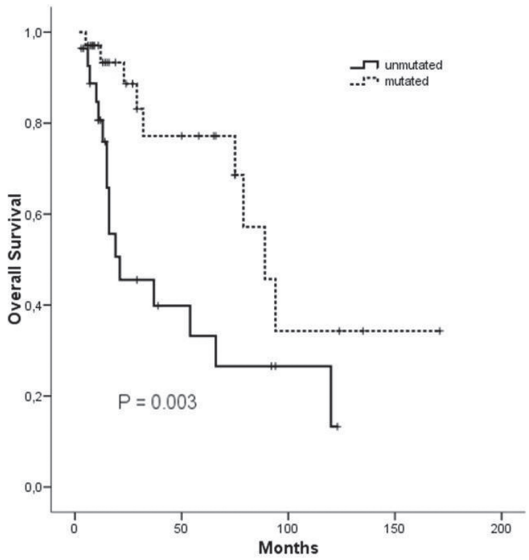

Figure 4. OS according to VH mutation status analysis results.

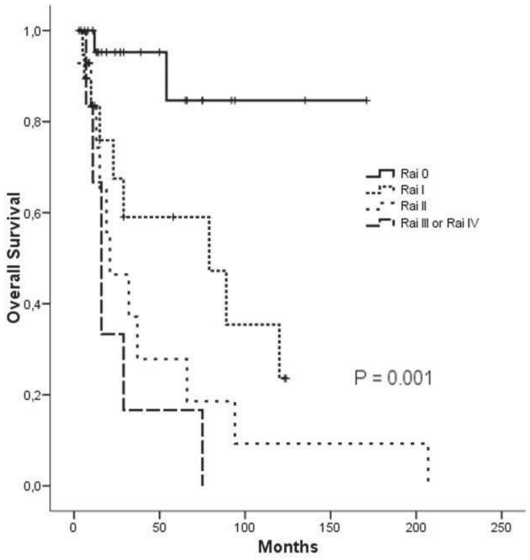

Figure 5. OS according to Rai stage.

Table V. Patients with I-FISH finding complete or partial IgV_H deletion.

Sex/age at dg.	Progression?	Deletion %	Infiltrate	Additional chromosomal aberrations	Mutated?	Germ line VH gene
complete deletion:						
M/64	0	81	93%	del(13)(q14)	0	NA
M/59	0	28	80%	del(13)(q14)	1	VH3-66
F/58	0	52.5	98%	del(13)(q14), del(17)(p13.1)	0	VH3-72
M/65	0	24	51%	del(13)(q14)	0	VH1-69
M/59	1	75	NA	del(13)(q14), del(11)(q22.3)	0	NA
M/55	NA	6.5	NA	del(13)(q14), del(11)(q22.3)	NA	NA
F/60	1	25.5	94%	del(13)(q14)	1	VH3-23
M/80	NA	60.5	NA	trisomy 12, del(11)(q22.3)	0	biclonal (VH1-69, VH4-61)
F/56	NA	51.5	NA	del(13)(q14)	NA	NA
F/57	0	78	96%	del(13)(q14), del(11)(q22.3)	0	VH1-69
F/70	1	80	50%	del(13)(q14), del(17)(p13.1)	1	VH3-15
F/80	0	70	40%	del(13)(q14)	1	VH3-72
F83	1	72.5	98%	no	0	VH1-58
M/63	0	12.5	50%	del(13)(q14)	1	biclonal (VH3-13, VH4-61)
M/57	1	88	94%	del(13)(q14), del(11)(q22.3)	0	VH-69
partial deletion:						
M/69	1	50.5	61%	del(13)(q14)	0	NA
F/54	NA	64.5	NA	del(13)(q14)	NA	NA
M/74	NA	100	NA	del(11)(q22.3)	0	VH1-69
F/75	1	95	88%	trisomy 12, del(11)(q22.3)	0	VH1-58
M/67	NA	85.5	NA	del(13)(q14)	NA	NA
M/73	1	90.5	80%	del(13)(q14), del(11)(q22.3)	0	VH1-69
M/82	NA	28	NA	trisomy 12, del(13)(q14), del(11)(q22.3)	NA	NA
M/82	0	34	89%	del(13)(q14), del(11)(q22.3)	1	VH4-31
F/45	NA	3.5	NA	del(13)(q14)	0	biclonal (VH3-30, VH5-51)
M/77	0	71.5	89%	del(13)(q14), del(17)(p13.1)	0	VH1-2
M/63	1	16	66%	del(11)(q22.3)	0	NA
F/57	0	62.5	87%	del(13)(q14)	1	NA
F/73	1	84.5	98%	no	1	NA
F/78	1	66.5	70%	del(17)(p13.1)	not identified	NA

deletion % – percentage of cells with complete or partial deletion detected by I-FISH; infiltrate % – percentage of malignant B lymphocytes detected by flow cytometry; NA – not available

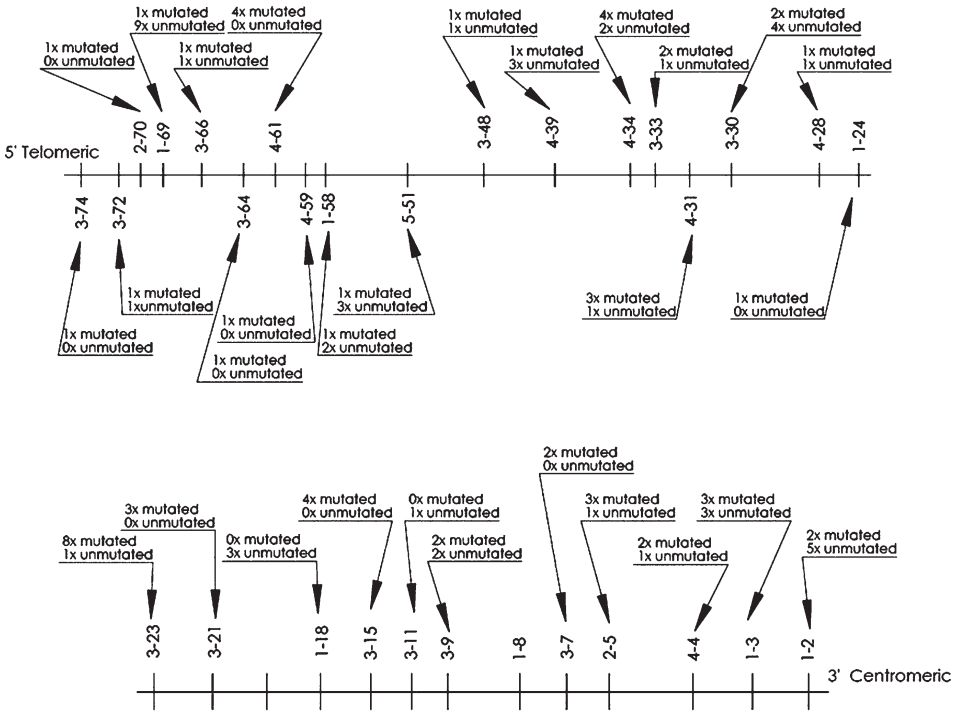

Figure 6. Scheme of variable segment of immunoglobulin heavy chain at 14q32.33 with localization of particular VH genes and their distribution together with mutation status results within presented cohort.

ined the most frequent genomic changes with interphase FISH as the conventional cytogenetic analysis is hampered by low number of dividing leukemic cells. I-FISH revealed chromosomal changes in 82% given by the extent of regions examined.

In accordance with previously reported studies, we have proved deletion of *13q14* to be the most common chromosomal abnormality (65%) with favorable prognostic impact (Figure 3).

Our results showed neither statistical significance (due to the small sample size) of trisomy *12,* found in 12% of cases, nor the adverse prognostic power described by some authors [21, 22]. Interestingly, statistical method Pearson´s logistic regression displayed correlation between trisomy *12* and age at diagnosis over 60 (data not presented), however, different authors suggest the contrary [23]. Nevertheless, *+12* has been repeatedly proposed to be a secondary event acquired through the course of the disease [24, 25].

We also proved negative prognostic impact of gene p53 deletion (detected in 17% of cases) and deletion of ATM (15%) especially within the subgroup with unmutated IgV_H genes (own observation). With regard to the fact that ATM protein is involved in dephosphorylation of protein p53 and thus defects of ATM protein can result in p53 inactivation [26], we have tested these two deletions as a derived variable (presence of either one or both deletions) using Kaplan-Meyer method which indicated significant differences between the survival curves (Figure 2).

The most problematic is the interpretation of IgH aberrations that were found in 31% of patients taking part in presented study. Though the total number of cases with IgH abnormal signal pattern was 45, once subdivided into four groups (deletion of variable segment of IgH gene, partial deletion of variable segment of IgH gene, deletion of 3´segment flanking IgH gene and split of the probe signals indicating translocation), these subgroups became statistically unrelevant due to low number of observations (Table II). Nevertheless, deletion of 3´segment flanking IgH gene is considered to have probably an intense negative prognostic impact but more cases with this finding need to be evaluated. The rearrangement of IgH gene complex indicating translocation occurred in 11 (24%) patients, thus we systematically continue in searching for translocation partner which is to be the aim of our prospective study.

Concerning the IgH deletions, Wlodarska et al. [27] proposes that the cryptic losses of various portions of IgH gene detected by FISH strictly correlate with localization of the germ line VH gene, indicating that they are not oncogenic but they reflect physiological events accompanying somatic V-D-J assembly taking place in the process of B-lymphocyte maturation in the bone marrow.

Consequently, we have decided to compare the localization of the VH gene used in the germ line (found by VH

sequencing) with the findings detected by I-FISH analysis using the LSI IGH probe. Mutation analysis data and VH gene identified in the germ line were available in 115 patients. Figure 6 shows IgV_H segment, localization of particular VH genes and their distribution together with mutation status results within presented cohort. We have not included the cases where result of mutation analysis was "germ line IgVH gene not identified" and "biclonal" (i.e. where two different VH genes were identified).

In compliance with above mentioned proposal, we expected that the most distally located used VH genes (e.g. VH3-74, VH3-72, VH1-69) will correlate with the LSI IGH I-FISH finding of complete or partial deletion of variable segment regardless of mutated or unmutated VH gene status. Surprisingly we proved this in some patients, however in the majority of cases, I-FISH with LSI IGH probe produced normal signal pattern and conversely, when used VH gene was located in the most proximal part of V segment (thus we supposed unidentifiable deletion i.e. normal I-FISH result) obvious deletions of IgV_H gene were detected in some cases. The localization of VH genes in patients with variable segment complete or partial deletion is listed in Table V. For these above mentioned reasons, we concluded that the putative origin and prognostic impact of IgV_H segment deletions remain unclear.

The strongest prognostic factor proved was mutation status. Our study also confirmed recently published statement that the patients with a VH1-69 usage in germ line tend to have predominantly unmutated status with an adverse prognostic impact [28] as of 10 patients 9 had unmutated IgV_H1-69 gene (Figure 6).

FISH screening of patients with B-CLL became important for risk stratification in addition to clinical, morphological, molecular and imunophenotype examinations. This study aspired to contribute to clarification of prognostic significance of the recurrent IgH gene complex deletions. Although deletions of 3´segment flanking IgH gene complex are supposed to have an adverse prognostic impact and the genetic background of variable segment deletions is believed to be most probably physiological, we assumed a detailed mapping of the 14q32.33 region is inevitable to unravel these mysteries.

This study was supported by grants MSM 0021620808, IGA MZCR 9244-3, MSM LC 535 and MZO 0064165.

References

[1] DAMESHEK W. Chronic lymphocytic leukemia – an accumulative disease of immunologically incompetent lymphocytes. Blood 1967; 29: 566–84.

[2] PATEL DV, RAI KR. Chronic lymphocytic leukemia. In: Hoffman R, Benz EJ, Shattil SJ, et al., eds. Hematology, principles and practice. 4th ed. Philadelphia: Elsevier Churchill Livingstone, 2005: 1437–54.

[3] CHIORAZZI N, RAI KR, FERRARINI M. Chronic lymphocytic leukemia. N Engl J Med 2005; 352: 804–15.

[4] RAI KR, SAWITZKY A, CRONKITE EP et al. Clinical staging of chronic lymphocytic leukemia. Blood 1975; 46: 219–234.

[5] BINET JL, LEPORRIER M, DIGHIERO G et al. A clinical staging system for chronic lymphocytic leukemia. Cancer 1977; 40: 855–864.

[6] CMUNT E, TRNENY M, KARBAN J et al. The risk factors analysis in 248 patients with B-cell chronic lymphocytic leukemia at diagnosis [in Czech]. Trans Hemat dnes 2006; 12: 232–239.

[7] DÖHNER H, STILGENBAUER S, JAMES MR et al. 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood 1997; 89: 2516–22.

[8] DÖHNER H, STILGENBAUER S, BENNER A et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 2000; 343: 1910–6.

[9] SHANAFELT TD, WITZIG TE, FINK SR et al. Prospective evaluation of clonal evolution during long-term follow-up of patients with untreated early-stage chronic lymphocytic leukemia. J Clin Oncol 2006; 24: 4634–41.

[10] DEWALD GW, BROCKMAN SR, PATERNOSTER SF et al. Chromosome anomalies detected by fluorescence in situ hybridization: correlation with significant biological features in B-cell chronic lymphocytic leukemia. Br J Haematol 2003; 121: 287–95.

[11] SINDELAROVA L, MICHALOVA K, ZEMANOVA Z et al. Incidence of chromosomal anomalies detected with FISH and their clinical correlations in B-chronic lymphocytic leukemia. Cancer Genet Cytogenet 2005; 160: 27–34.

[12] MITELMAN F, JOHANSSON B, MERTENS F. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 2007; 7: 233–45.

[13] NOWAKOWSKI GS, DEWALD GW, HOYER JD et al. Interphase fluorescence in situ hybridization with an IGH probe is important in the evaluation of patients with a clinical diagnosis of chronic lymphocytic leukaemia. Br J Haematol 2005; 130: 36–42.

[14] CHESON BD, BENNETT JM, GREVER M et al. National Cancer Institute – sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 1996; 87: 4990–4997.

[15] HAMBLIN TJ, DAVIS Z, GARDINER A et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999; 94: 1848–54.

[16] STILGENBAUER S, BULLINGER L, LICHTER P et al. German CLL Study Group (GCLLSG). Genetics of chronic lymphocytic leukemia: genomic aberrations and V(H) gene mutation status in pathogenesis and clinical course. Leukemia 2002; 16: 993–1007.

[17] SCHWARZ J, MIKULENKOVA D, CERMAKOVA M et al. Prognostic relevance of the FAB morphological criteria in chronic lymphocytic leukemia: correlations with IgVH gene mutational status and other prognostic markers. Neoplasma 2006; 53: 219–25.

[18] A. FIELD et al. Discovering Statistics Using SPSS. 2nd ed., Sage Publications Ltd.,Thousand Oaks, 2005.

[19] J. F. HAIR, B. BLACK, B. BABIN, R. E. ANDERSON, R. L. Tatham. Multivariate Data Analysis. 6th ed., Prentice Hall, Upper Saddle River, 2005.

[20] D. W. HOSMER, S. LEMESHOW. Applied Survival Analysis. John Wiley and Sons, New York, 1999.

[21] JULIUSSON G, GAHRTON G. Chromosome aberrations in B-cell chronic lymphocytic leukemia. Pathogenetic and clinical implications. Cancer Genet Cytogenet 1990; 45: 143–60.

[22] HAMBLIN TJ. Trisomy 12 in CLL revisited. Leuk Res 1997; 21: 1025–6.

[23] RIPOLLES L, ORTEGA M, ORTUNO F et al. Genetic abnormalities and clinical outcome in chronic lymphocytic leukemia. Cancer Genet Cytogenet 2006; 171: 57–64.

[24] DEWALD GW, BROCKMAN SR, PATERNOSTER SF et al. Chromosome in situ hybridization: correlation with significant features of B-cell chronic lymphocytic leukaemia. Br J Haematol 2003; 121: 287–95.

[25] ATHANASIADOU A, STAMATOPOULOS K, TSOMPANAKOU A et al. Clinical, immunophenotypic, and molecular profiling of trisomy 12 in chronic lymphocytic leukemia and comparison with other karyotypic subgroups defined by cytogenetic analysis. Cancer Genet Cytogenet 2006; 168: 109–19.

[26] PETTITT AR, SHERRINGTON PD, STEWART G et al. p53 dysfunction in B-cell chronic lymphocytic leukemia: inactivation of ATM as an alternative to TP53 mutation. Blood 2001; 98: 814–22.

[27] WLODARSKA I, MATTHEWS C, VEYT E et al. Telomeric IGH losses detectable by fluorescence in situ hybridization in chronic lymphocytic leukemia reflect somatic VH recombination events. J Mol Diagn 2007; 9:47–54.

[28] PANOVSKA-STAVRIDIS I, IVANOVSKI M, SILJANOVSKI N et al. Chronic lymphocytic leukemia patients with a V 1-69 gene rearrangement do not have inferior survival with respect to patients that express other unmutated VH genes. Leuk Res 2007; 31:245–8.